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Sediment and Vegetation as Reservoirs of *Vibrio vulnificus* in the Tampa Bay Estuary and Gulf of Mexico

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The opportunistic pathogen *Vibrio vulnificus* occurs naturally in estuarine habitats and is readily cultured from water and oysters under warm conditions but infrequently at ambient conditions of <15°C. The presence of *V. vulnificus* in other habitats, such as sediments and aquatic vegetation, has been explored much less frequently. This study investigated the ecology of *V. vulnificus* in water by culture and quantitative PCR (qPCR) and in sediment, oysters, and aquatic vegetation by culture. *V. vulnificus* samples were taken from five sites around Tampa Bay, FL. Levels determined by qPCR and culture were significantly correlated ($P = 0.0006$; $r = 0.352$); however, *V. vulnificus* was detected significantly more frequently by qPCR (85% of all samples) compared to culture (43%). Culturable *V. vulnificus* bacteria were recovered most frequently from oyster samples (70%), followed by vegetation and sediment (~50%) and water (43%). Water temperature, which ranged from 18.5 to 33.4°C, was positively correlated with *V. vulnificus* concentrations in all matrices but sediments. Salinity, which ranged from 1 to 35 ppt, was negatively correlated with *V. vulnificus* levels in water and sediments but not in other matrices. Significant interaction effects between matrix and temperature support the hypothesis that temperature affects *V. vulnificus* concentrations differently in different matrices and that sediment habitats may serve as seasonal reservoirs for *V. vulnificus*. *V. vulnificus* levels in vegetation have not been previously measured and reveal an additional habitat for this autochthonous estuarine bacterium.

Vibrio vulnificus is an opportunistic human pathogen that causes gastroenteritis and rapidly fulminating, frequently fatal septicemia (1). Wounds may become infected by *V. vulnificus* via contact with seawater, fish, or oysters, which may result in necrotizing fasciitis leading to limb amputation (2). Although certain medical conditions act as predisposing factors for infectivity (1, 3), the bacterium remains a threat to shellfish consumers, beachgoers, and those who engage in fishing activities.

Vibrio vulnificus is found in estuaries of tropical and temperate waters where it faces frequently changing aspects of its environment (4, 5). Culturable concentrations of *V. vulnificus* typically decline with decreasing water temperature (6, 7). The highest concentrations are observed during warm months, and the bacterium may enter a viable but not culturable (VBNC) state when water temperature falls below ~15°C (8–10). Salinity influences culturable concentrations of *V. vulnificus*, as the highest levels are generally observed at salinities ranging from 5 to 25‰ (6, 11), and *in vitro* experiments found that optimal growth occurs in salinities of between 5 and 30‰ (12).

The marked reduction or absence of culturable *V. vulnificus* cells in the environment during the winter, followed by its reappearance as waters warm (4, 5, 7, 13), suggests that physiological adaptations exist for overwintering. The majority of studies that explored the temporal distribution of *V. vulnificus* analyzed only water and/or oyster matrices (4, 6, 10, 13–15). However, the densities of *V. vulnificus* were often greater in sediments and floc compared to the water column in Apalachicola Bay, FL (16, 17). More recently, culturable *V. vulnificus* cells were isolated from sediment samples collected during cold months along the Mississippi Gulf Coast when they could not be isolated from water or oysters (5). No previous studies have determined whether *V. vulnificus* can be detected in submerged aquatic vegetation and whether temperatures affect concentrations. Studies have shown positive correlations between *V. vulnificus* and abundance of marine particulates, including decapods, copepods, and diatoms (18), but no significant relationships with temperature have been determined in habitats other than water.

In the present study, water, sediments, oysters, and submerged aquatic vegetation (SAV) were collected from marine and estuarine sites in the Tampa Bay portion of the Gulf of Mexico. We hypothesized that the bacterium resides in sediments and SAV, which provide habitats for overwintering. Furthermore, this study compared two methods of isolation of *V. vulnificus* targets from the environment—the most probable number (MPN) culture-dependent procedure involving overnight enrichment in alkaline peptone water (APW), and a culture-independent quantitative PCR (qPCR) assay targeting the *vvhA* hemolysin gene, detecting cells that could not be recovered by culture, such as those in a VBNC state.

MATERIALS AND METHODS

Site description, sample collection, and preparation. Five sites were selected within the Tampa Bay area: two tidally influenced streams with low to intermediate salinities (Bullfrog Creek [BC], 27°50′17″N, 82°22′55″W; Upper Tampa [UT], 28°0′47″N, 82°38′1″W), two marine beaches (Sun-

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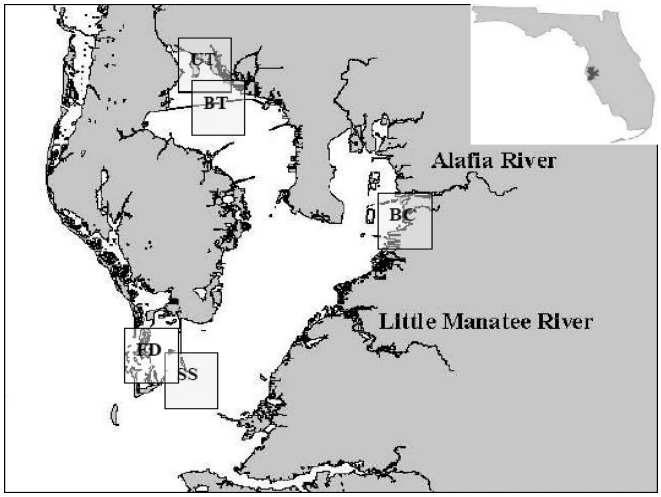

FIG 1 Location of the sampling sites in this study: Bullfrog Creek (BC), Sunshine Skyway (SS), Fort DeSoto (FD), Ben T. Davis (BT), and Upper Tampa (UT).

shine Skyway [SS], 27°39′28″N, 82°40′35″W; Fort DeSoto [FD], 27°38′7″N, 82°43′6″W), and an estuarine beach (Ben T. Davis [BT], 27°58′14″N, 82°34′44″) (Fig. 1). Both BC and BT are frequently affected by stormwater runoff, and BC is affected by failing septic systems. Shellfishing was prohibited at both sites because of chronically elevated fecal coliform concentrations (19). BC is positioned within a residential area characterized by mobile home communities with onsite sewage treatment and disposal systems (OSTDS). BT is a popular city beach frequently used for bathing and recreational fishing. Shellfishing was permitted at both SS and FD for the duration of the study. Due to its remote location, SS receives little human traffic, which is generally limited to recreational fishing. FD is located inside a campground that is connected to the city sewer system. UT is positioned inside a state park. Human activities at this site, including fishing and shellfishing, are limited and controlled by park regulations.

Samples were collected every month from May 2011 to April 2012 ($n = 12$). One sample of each matrix was collected per site. All samples were collected and processed on the same day. The physiochemical parameters measured *in situ* included temperature, salinity, pH, and dissolved oxygen. Water samples were collected into sterile 1-liter bottles, sediments were gathered into sterile 50-ml tubes, and oysters and submerged aquatic vegetation were placed in plastic bags. Samples from each matrix were collected in close proximity to each other at each site. Sediment and vegetation were collected randomly and were not distinctly variable among sites, although further taxonomy or soil characterization was not conducted. Sediment was collected within 5 cm of the surface. Vegetation samples represented a mixture of common species in the ecosystems from which they were collected, including hydrilla at freshwater sites and common seagrasses from marine and estuarine sites. The most common seagrasses in this region include turtle grass (*Thalassia testudinum*), manatee grass (*Syringodium filliforme*), and shoal grass (*Haoldule wrightii*). Samples were processed within 6 h of sampling.

Isolation of *Vibrio vulnificus*. The three-tube most probable number (MPN) method described in the Food and Drug Administration's *Bacteriological Analytical Manual* (FDA BAM) was used to estimate *V. vulnificus* concentrations, (20). A control *V. vulnificus* strain (CMCP6) was processed monthly through all culture steps as a positive control. Sediments and vegetation (20 g) were first diluted 1:1 (wet wt/vol) in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 0.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) and then hand-shaken for 2 min to release bacteria attached to particles (21). Oysters were dissected aseptically and combined into 40- to 50-g composites. Composites were diluted 1:1 in PBS and homogenized by blending for 1 min in a Waring blender (Waring Products, Torrington, CT). Samples from all matrices were serially diluted and enriched overnight at 37°C in alkaline peptone water (APW) (22). Enriched samples were then streaked on CPC+ medium (23), and putative *V. vulnificus* colonies (flat, round, yellow colonies 1 to 2 mm in diameter) were confirmed by conventional PCR targeting the *vvhA* gene (24). Final concentrations were reported as MPN · 100 ml^{-1} (water) or MPN · 100 g^{-1} wet weight (sediment, oysters, and vegetation). The limits of detection for water were 30 MPN/100 ml and 60 MPN/100 g for sediment, oysters, and vegetation. Serial dilutions were processed to account for potential exceedance, and in no case did the level of *V. vulnificus* exceed the upper detection limit of 11,000 CFU per 100 ml or per 100 g.

DNA extraction and PCR analyses. A conventional PCR assay was carried on all putative *V. vulnificus* targets following the FDA-recommended procedure (24). Template DNA for each conventional PCR was obtained by colony pick from CPC+ agar plate with a sterile pipette tip. The cell biomass was placed directly into the reaction tubes. DNA from *V. vulnificus* strains CMCP6 (a clinical isolate from Chonnam National University Hospital, South Korea), 9067-96 (25), and ATCC 27562 were used as positive controls. PCR products were visualized by electrophoresis on a 2% agarose gel.

Five hundred milliliters of water per sample was filtered through 0.45-μm-pore nitrocellulose filters for qPCR. Filters were stored at −20°C until processed. DNA from filters was extracted using the MoBio PowerSoil DNA isolation kit (MoBio, Carlsbad, CA) following the manufacturer's instructions. A previously published qPCR method (26) targeting the *V. vulnificus* hemolysin gene (*vvhA*) was utilized for all water samples. The protocol was modified from the use of Sybr green to Bryt green GoTaq master mix (Promega, Madison, WI). The results obtained using Bryt green have been previously found comparable to those utilizing Sybr green (12). All reactions were run in duplicate, and standard curves were included with each run. All samples were tested for inhibition in the qPCR assay by adding a known amount of salmon sperm DNA to reaction mixtures with purified template DNA. The control DNA was quantified by qPCR, and threshold cycle (C_T) values more than 1 greater than expected denoted inhibition. No inhibition was found in any purified DNA sample. Genomic DNA extracted from a CMCP6 strain served as a standard curve template for *V. vulnificus* assay. All reactions were performed using the Applied Biosystems 7500 real-time PCR system (Carlsbad, CA). All qPCR results are reported as gene copies · 100^{-1} ml.

Statistical analyses. All bacterial concentrations were $\log_{10}$ transformed prior to analyses. Due to the frequent lack of detection of culturable *V. vulnificus* organisms, one-half the detection limit was used for "non-detect" observations (27). A general linear model with a full factorial design was performed with univariate tests for significance with sigma-restricted parameterization using Statistica version 12 for Windows (StatSoft Software, Tulsa, OK). Chi-square analyses for frequency of detection by matrix and Pearson's correlations for relationships between variables were conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was assessed at $\alpha = 0.05$.

RESULTS

***V. vulnificus* detection.** The frequency of *V. vulnificus* detection in the Tampa Bay area sites (Fig. 1) varied among the matrices, sites, and methods of detection (culture versus qPCR) (Table 1). The frequency of detection of *V. vulnificus* by culture was significantly different by matrix (chi-square test, $P < 0.0001$). *V. vulnificus* was cultured from 43, 50, 70, and 52% of sampling events from water, sediments, oysters, and aquatic vegetation, respectively, from all sites combined ($n = 60$ samples per matrix). Figure 2 shows the proportion of all *V. vulnificus* isolates each month that originated from each matrix and water temperature. In many months, the majority of isolates were obtained from oysters, but

TABLE 1 Frequency of *V. vulnificus* detection by culture and qPCR per site and matrix

Site/matrix	Frequency of detection (%)[a]					
	Culture					qPCR
	Water	Sediment	Oysters	Vegetation	Combined[b]	(water)
BC	75	92	83	67	79	92
FD	42	33	50	50	44	92
SS	17	8	50	25	25	67
BT	50	58	92	58	65	83
UT	33	58	75	58	56	92
All sites combined ($n = 60$)	43	50	70	52	54	85

[a] Frequency is expressed as the percentage of positive detections ($n = 12$ sample events/site).
[b] The "Combined" column includes culture data from all matrices.

sediments accounted for a major proportion of the isolates in many sites over the sampling period. The frequency of *V. vulnificus* detection by culture was also significantly different by site, being highest at BC (79%) and lowest at SS (25%) (chi-square test, $P < 0.0001$) (Table 1). The frequency of detection of *V. vulnificus* in water by qPCR using the *vvhA* gene (85%) was greater than that of detection in water by culture (43%) (chi-square test, $P < 0.0001$).

***V. vulnificus* concentrations.** *Vibrio vulnificus* was cultured from all matrices (water, sediments, oysters, and vegetation), with geometric mean concentrations of 21, 38, 82, and 34 MPN · 100 ml^{-1} (water) or MPN · 100 g^{-1} wet weight (sediment, oysters, and vegetation), respectively (see Table S1 in the supplemental material). Table S1 also contains physicochemical parameters. Culturable concentrations were significantly different based on matrix (analysis of variance [ANOVA], $P = 0.0062$), with the highest concentrations found in oysters. Levels in oysters were significantly different from those in water and vegetation (Tukey's post-test; $P < 0.05$) but not sediment. A significant interaction between mean water temperature and matrix was observed ($P = 0.0056$), which was due to the correlation of *V. vulnificus* levels in all matrices except for sediment (Table 2). qPCR measurements of *V. vulnificus* concentrations were significantly correlated with culturable concentrations ($P = 0.0006$; $r = 0.352$) and 2.7 $\log_{10}$ greater, on average, than culture-dependent concentrations (see Table S1).

Correlations among *V. vulnificus* and physiochemical factors. Significant positive correlations between the levels of culturable *V. vulnificus* and water temperature, which ranged from 18.5 to 33.4°C (see Table S1 in the supplemental material), were observed for water, oysters, and vegetation using the mean water temperature at all sites on a given date, while no significant correlations were observed in sediment (Table 2). When site-specific temperatures were used in the analysis, only levels in vegetation were significantly correlated with temperature (Table 2). *V. vulnificus* levels determined by qPCR (water samples only) correlated positively with site-specific and average water temperatures (Fig. 3A) (*P* values of 0.0185 and 0.012, respectively). Figure 3B shows the similar correlation of culturable levels of *V. vulnificus* with water temperature. Peak *V. vulnificus* levels in all matrices occurred in June, when water temperatures were highest (see Table S2 in the supplemental material). Negative correlations between *V. vulnificus* and salinity were significant in water by qPCR ($P < 0.0001$; $r = -0.547$) and in sediment by culture ($P = 0.0004$; $r = -0.365$) (see Fig. S1A and S1B in the supplemental material), but there were no significant correlations with salinity in water ($P = 0.229$), oysters ($P = 0.167$), or vegetation ($P = 0.086$) by culture.

DISCUSSION

This study shows that sediments and vegetation are supportive habitats for culturable *V. vulnificus*. One recent study reported the presence of culturable *V. vulnificus* cells in sediments during a winter in northern Gulf of Mexico, while the concentrations of the bacterium fell below the limit of detection in other matrices (5). Reservoirs of *V. vulnificus* in the aquatic environment may sustain populations of viable *V. vulnificus* cells through exposure to non-ideal environmental conditions, such as low temperatures. Nearshore Gulf of Mexico water temperatures at the sites in this study did not go lower than 18.5°C (in January at site SS); therefore, conditions were not appropriate for testing hypotheses about the relative importance of the environmental matrices in cold temperatures. Tampa area water temperatures can be as low as 11°C in winter months, which is much lower than the minimum observed in this 1-year study; therefore, further data collection could shed more light on this subject. Nonetheless, the lack of correlation of *V. vulnificus* in sediments with water temperature suggests that bacterial concentrations in sediments are not as responsive to temperature as those in the other matrices sampled here. Attachment to sediment particles and biofilm formation may contribute to this differential survival (28). While patchiness of environmental matrices can impact results based on collection regimens, the sampling plan for collecting both sediment and vegetation in this study was comprehensive and geographically distributed. Vegetation showed a significant correlation with temperature, while sediment did not: patchiness may be an issue but is not the sole driver of these results.

This study was among the first to investigate the temporal distribution of *V. vulnificus* on submerged aquatic vegetation. *V. vulnificus* was detected in vegetation and sediment in approximately 50% of samples. However, positive correlation of *V. vulnificus* concentrations with temperature was determined for vegetation but not sediments. *V. vulnificus* was previously isolated from seaweed in Japan during periods of cold temperature when no culturable cells could be obtained from the water column (29). SAV may provide the bacterium with surfaces for attachment and nutrients. Submerged macrophytes have been shown to increase

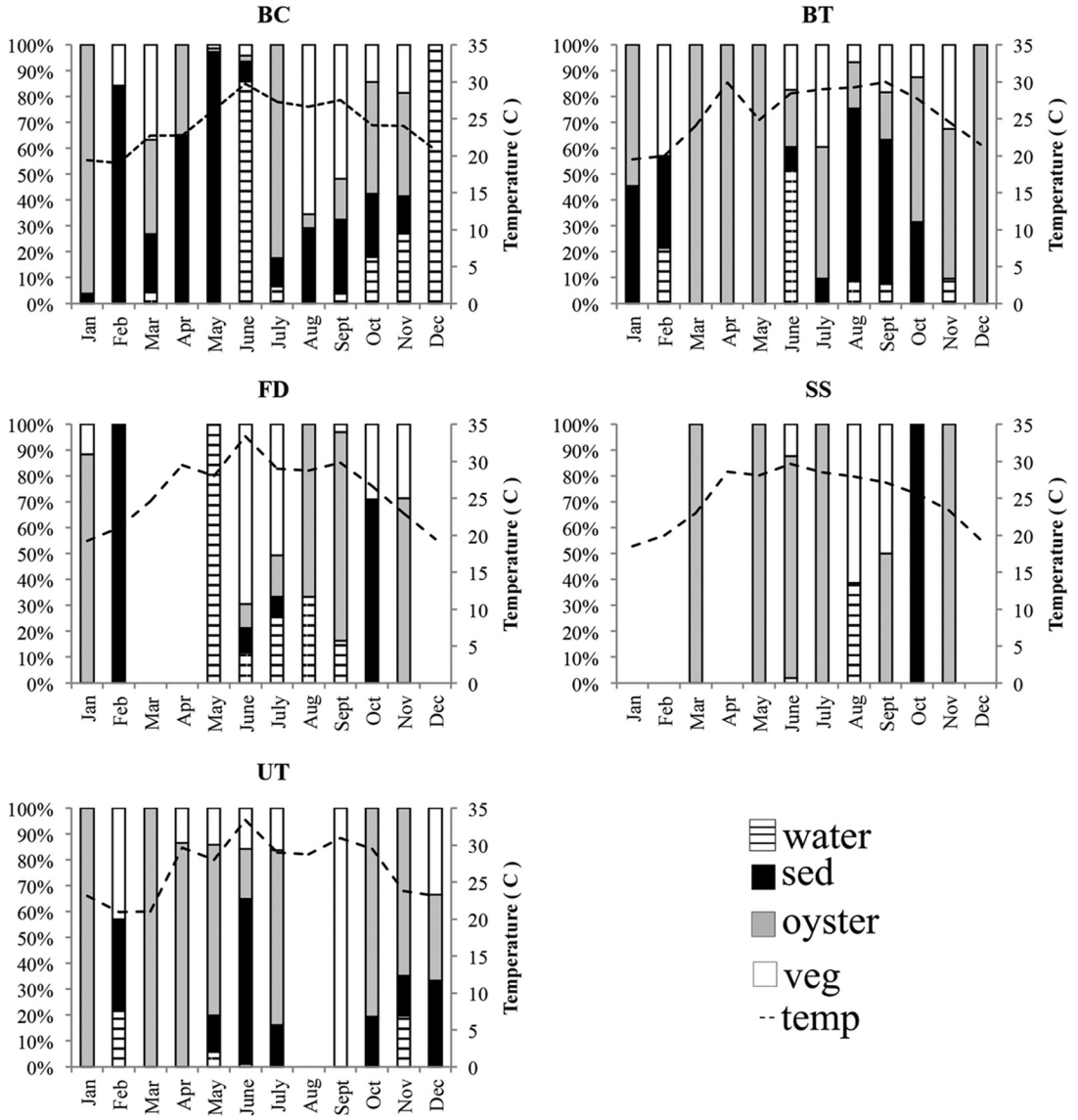

FIG 2 Proportion of *V. vulnificus* isolates by matrix, when detected, and temperature as measured at each site. sed, sediment; veg, vegetation; temp, temperature.

available carbon to surrounding sediments (30). Furthermore, in the case of *Escherichia coli*, the presence of SAV in the aquatic habitats was associated with increased *E. coli* concentrations in the water column following the efflux of attached cells (31); thus, SAV may lead to increases in *V. vulnificus* through similar processes.

TABLE 2 Correlation of culturable *V. vulnificus* concentrations with water temperature

Matrix	Correlation with[a]:			
	Site temp		Avg temp	
	P value	*r*	*P* value	*r*
Water	0.127	0.199	**0.043**	0.262
Sediment	0.717	0.048	0.354	0.122
Oysters	0.097	0.295	**0.048**	0.328
Vegetation	**0.022**	0.216	**0.007**	0.256

[a] Statistically significant *P* values are in boldface, and correlation coefficients (Pearson's *r*) are reported.

The highest frequency of detection and greatest mean concentrations of culturable *V. vulnificus* were found in oysters. *V. vulnificus* levels in all matrices were greatest in June, when water temperatures were highest. Results for oysters and water are consistent with those from other Gulf of Mexico studies, which have observed the greatest *V. vulnificus* levels between May and October (6) and lows between November and March (5, 6).

The lowest frequency of detection of *V. vulnificus* by culture was noted in the water column; however, *V. vulnificus* was almost always detected by qPCR in this matrix, even when the bacterium could not be isolated by culture. Interestingly, both methods of detection, culture and qPCR, followed the same temporal pattern and a significant positive correlation was found between the bacterial levels detected by both methods. It is, therefore, likely that a large proportion of the qPCR targets were viable but not culturable (VBNC). The VBNC state, a possible survival strategy for many species, several of which are human pathogens, has been well documented in *V. vulnificus* (9, 32, 33). The instances when *V.*

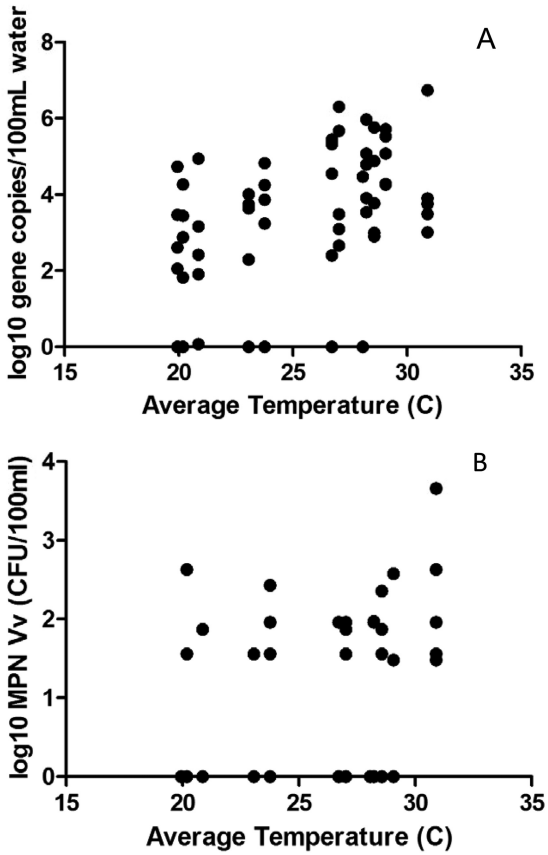

FIG 3 Correlations of *V. vulnificus* levels in water with average water temperature. (A) qPCR measurements of *V. vulnificus vvhA* gene concentrations ($n = 60$; $P = 0.012$; $r = 0.321$); (B) culturable *V. vulnificus* (Vv) ($n = 60$; $P = 0.043$; $r = 0.262$).

vulnificus was not detected by culture but was detected by qPCR during warmer months (e.g., August at UT) may be due to competition with other bacteria during enrichment culture. Other *Vibrio* spp. have been shown to outcompete *V. vulnificus* in warm, estuarine waters (34), specifically in the case of *Vibrio sinaloensis* (35).

Sediments make up a large proportion of total available biological matrices across a coastal ecosystem and should be considered potentially important sources and sinks of waterborne pathogens. Water, sediment, and vegetation had lower culturable *V. vulnificus* concentrations than oysters, but they provide the greatest amount of habitat in terms of proportional available surface area in the ecosystem. Proportions of bacteria per matrix can also change on a relatively short time scale, based on the example of enterococci (36), and this study suggests that environmental conditions (temperature) can contribute to shifts in dominant habitats of *V. vulnificus*. Thus, it is important to consider the relative contributions rather than absolute measurements of concentrations in different matrices and to consider complexities regarding physiochemical characteristics of an area.

The roles of sediment and vegetation as habitats and potential reservoirs of *V. vulnificus* merit further investigation considering the data obtained during this study. Studies conducted in waters that reach colder temperatures may well clarify the contribution of sediments to survival of this ubiquitous estuarine pathogen at suboptimal temperatures.

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